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Research Note

The nitric oxide specific scavenger carboxy-PTIO does not inhibit smoke stimulated germination of Grand Rapids lettuce seeds

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As part of a continued effort to understand the mechanism underlying smoke-stimulated seed germination, the effect of two nitric oxide (NO) donors N-tert-butyl-α-phenylnitrone (PBN) and sodium nitroprusside (SNP) on the germination of Grand Rapids lettuce seeds were investigated. Seeds were treated with solutions of PBN and SNP at concentrations from 10–1 000µm, and with a combination of 1:1 000 aqueous smoke solution and the NO specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium (c-PTIO). No enhanced germination was observed with the PBN and SNP treatments, and c-PTIO showed no inhibition of the germination stimulated by a 1:1 000 dilution of an aqueous smoke solution. These results suggest that factors, other than NO, are responsible for the enhanced germination of Grand Rapids lettuce seeds by aqueous smoke solutions.

The phenomenon of smoke-stimulated germination was first identified in 1990 by De Lange and Boucher. Since then, numerous studies have been conducted in attempts to elucidate the mechanism of smoke-stimulated germination, and to isolate compound(s) which are responsible for the action of smoke and aqueous smoke solutions on the seed germination of many species (Brown and Botha 2002). However, the active compound(s) and the mechanism whereby the germination of many seeds is stimulated remains unknown, although a number of hypotheses have been suggested (Brown and Van Staden 1997, Van Staden *et al.* 2000).

Nitric oxide (NO) and its related nitrogen oxides have been reported as stimulators of seed germination in a number of studies and it has been suggested that these nitrogenous compounds are responsible for the promotion of germination by smoke. Thanos and Rundel (1995) reported that nitrates stimulated the germination of two post-fire annuals, *Emmenanthe penduliflora* and *Phacelia grandiflora*, and concluded that nitrate was the principal factor for inducing the germination in these species. In another study, nitrogen oxides have been shown to induce 100% seed germination of *E. penduliflora* seeds in a manner similar to smoke (Keeley and Fotheringham 1997). NO_2 was more stimulatory than NO_x (NO + NO_2) and induced germination both directly and indirectly. Furthermore, it was estimated that wildfires generate large amounts of nitrogen oxides from the combustion of organic matter to trigger the germination of *E. penduliflora*. In a further study by Keeley and Fotheringham (1998) investigating the mechanism of smoke-induced seed germination, the post-fire flora of Californian chaparral species did not appear to be triggered by nitrate. However, NO_2 at concentrations present in biomass smoke was effective at inducing germination.

Recently, there has been much research on the role of NO in plant cells, which is a bioactive molecule in mammalian cells. NO is recognised to be an important signal and effector molecule during development and in adaptive plant responses, supporting the idea of NO as a versatile molecule with variable functions, as seen in animal systems (Pagnussat *et al.* 2002). For example, NO promotes seed germination and de-etiolation, and inhibits hypocotyl and internode elongation, all light-inducible responses in plants (Beligni and Lamattina 2000).

Beligni and Lamattina (2000) reported that the NO-donor sodium nitroprusside (SNP) at a concentration of 100µM promoted the germination of Grand Rapids lettuce seeds in the dark. The NO specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium (c-PTIO) was used in a number of studies to counteract the effect of NO and NO-donor compounds, and showed a marked inhibition of the SNP and *S*-nitroso-*N*-acetylpenicillamine (SNAP) stimulated germination of Grand Rapids lettuce seeds at a concentration of 100µM (Beligni and Lamattina 2000). In this same study, nitrite and nitrate, two NO-decomposition products, were ineffective in stimulation germination of Grand Rapids lettuce seeds. Similarly, the

NO releasing compounds SNP and SNAP stimulated the germination of *Paulownia tomentosa* seeds at concentrations between 100µM and 1 000µM (Giba *et al.* 1998).

An investigation was undertaken to determine the effect of N-tert-butyl-α-phenylnitrone (PBN) and SNP on the germination of Grand Rapids lettuce seeds, and to see if c-PTIO showed any inhibitory effect of smoke stimulated germination of Grand Rapids lettuce seeds.

Mature achenes (referred to as seeds) of *Lactuca sativa* L. cv. Grand Rapids (Peto Seed, Saticoy, USA) were stored in the dark at 4°C. All manipulations of seeds were carried out in the dark under a green 'safelight' (Drewes *et al.* 1995). Smoke solutions obtained from the dilution of an aqueous smoke extract produced in 1994 from burnt *Themeda triandra* material according to the method outlined by Baxter *et al*. (1994) were used throughout. A dilution of 1:1 000 (pH 4.7) has been established as the concentration giving optimal germination of Grand Rapids lettuce seeds.

For each treatment, four replicates of 25 seeds were incubated in the dark at 25°C for 24h. Seeds were placed in a 65mm plastic petri dish fitted with two sheets of Whatman No. 1 filter paper and moistened with 2ml of test solution. The chemicals N-tert-butyl-α-phenylnitrone (PBN) (Sigma) and sodium nitroprusside (SNP) (Sigma) were used as NO donors. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium (carboxy-PTIO potassium) (C-PTIO) (Sigma) was used as an NO scavenger. Seeds were treated with 10µM, 100µM and 1 000µM of PBN and SNP, and mixtures containing 1:1 000 smoke solution and 10µM, 100µM or 1 000µM c-PTIO. To determine any inhibition by the PBN and SNP test solutions (including 10mM), a set of seeds was exposed to 10min red light (660nm = 1.8µmol m^{-2} s^{-1}; PAR value of 26.4µmol m^{-2} s^{-1}) after 2h imbibition in the dark. Distilled water was used as a control, and a 1:1 000 dilution of the smoke solution was used as a standard treatment in each set of experiments. Each experiment was repeated twice.

Seeds treated with water alone showed minimal levels of germination in the dark (Table 1). Treatment of the lettuce seeds with PBN at a concentration of 10mM inhibited germination, even when treated with 10min of red light (results not shown). Treatment with SNP at 10mM did not inhibit germination of the red light-treated seeds, but the radicles appeared to be slightly discoloured and had shorter root hairs in comparison to the water control. At lower concentrations (10µM, 100µM and 100µM), both the PBN and SNP showed no inhibition of germination (red light treatment, results not shown), but did not enhance the germination of seeds incubated in the dark, contrary to the observations of Beligni and Lamattina (2000). It is possible that the use of different seed batches could account for these discrepancies in results. However, a treatment of 1:1 000 smoke solution stimulated germination above 80%, suggesting that factors in the smoke, other than NO may be responsible for the enhanced germination.

In combination with 1:1 000 aqueous smoke solution, c-PTIO did not show any inhibition of the stimulatory effect of the smoke. It was expected that the c-PTIO might show some inhibition of the enhanced germination due to the aqueous smoke solution if NO in the smoke was responsible for the germination.

Table 1: Effect of PBN, SNP, c-PTIO and an aqueous smoke solution on the germination of Grand Rapids lettuce seeds in the dark at 25°C

Treatment	Germination (%)[a]
H_2O	21.7.±.11.0
Smoke 1:1 000	86.6.±.11.1
10µm PBN	18.0.±.18.4
100µm PBN	14.7 ± 13.1
1 000µm PBN	12.0 ± 7.2
10µm SNP	10.5 ± 9.6
100µm SNP	14.0 ± 11.5
1 000µm SNP	12.5 ± 7.2
1:1 000 Smoke + 10µm c-PTIO	81.0 ± 11.3
1:1 000 Smoke + 100µm c-PTIO	80.0 ± 13.9
1:1 000 Smoke + 1 000µm c-PTIO	77.0 ± 19.1

[a] Germination percentages are the mean values ±SD of two independent experiments consisting of four replicates of 25 seeds

It is possible that traces of NO in smoke and smoke solutions do play a role in the signalling of germination, as seen in the number of species that do respond to treatments of NO-releasing and other nitrogenous compounds. However, the inability of the two NO-donors used to enhance germination, and the germination of seeds by aqueous smoke solution in the presence of c-PTIO, an NO scavenger, suggest that this is not the only factor present in smoke which is responsible for the germination. At present, the compound(s) responsible for the stimulatory action of smoke on seed germination and the mode of action remain unknown, and still require further research.

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